Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens

K. W. Lee,*† S. H. Lee,* H. S. Lillehoj,*[1] G. X. Li,* S. I. Jang,* U. S. Babu,‡ M. S. Park,* D. K. Kim,* E. P. Lillehoj,§ A. P. Neumann,# T. G. Rehberger,# and G. R. Siragusa#

*Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, USDA, Beltsville, MD 20705; †National Veterinary Research and Quarantine Service, Ministry for Food, Agriculture, Forestry and Fisheries, 480 Anyang 6-dong, Anyang City, Kyunggido 430-824, South Korea; ‡Immunobiology Branch, Division of Virulence Assessment, Center for Food Safety and Applied Nutrition, US Food and Drug Administration, 8301 Muirkirk Rd., Laurel, MD 20708; §Department of Pediatrics, University of Maryland School of Medicine, Baltimore 21201; and #Danisco, W227 N752 Westmound Drive, Waukesha, WI 53186

ABSTRACT This study was conducted to compare growth performance, gut morphometry, and parameters of local and systemic immunity in broiler chickens fed for 22 consecutive days with a diet supplemented with *Bacillus* spp. as direct-fed microbials (DFM), a commercial product incorporating 3 DFM, or a non-supplemented diet. Direct-fed microbials did not significantly modify BW gain and most failed to affect serum antibody levels in response to immunization with a recombinant *Eimeria* protein. However, altered intestinal morphometric measurements were readily apparent in DFM-fed chickens as revealed by increased villus height and crypt depth compared with non-DFM-fed controls. In addition, serum levels of α-1-acid glycoprotein as an inflammatory marker were reduced in DFM-fed birds, whereas splenic lymphocyte proliferation, intestine intraepithelial lymphocyte subpopulations, and cytokine mRNA levels in intraepithelial lymphocytes were increased, decreased, or unchanged compared with controls depending on the DFM used. These results provide a rational scientific basis for future studies to investigate DFM as immunomodulating agents to enhance host protective immunity against enteric pathogens in broiler chickens.

Key words: chicken, direct-fed microbial, immune response, growth performance, intestinal morphometry

2010 Poultry Science 89:203–216
doi:10.3382/ps.2009-00418

INTRODUCTION

Enteric diseases in commercial poultry contribute to losses in productivity, increased mortality, and contamination of products for human consumption (Dekich, 1998; Patterson and Burkholder, 2003). Among the diseases of high concern are those due to *Clostridium*-related infections (such as gangrenous dermatitis or turkey cellulitis in poults and necrotic enteritis), enteritis of unknown etiologies, and colibacillosis (Smith and Helm, 2008). A variety of different approaches to enhance intestinal immunity have been explored to reduce the incidence of poultry enteric disorders (Reid and Friendship, 2002; Callaway et al., 2008). Among these, direct-fed microbials (**DFM**), or probiotics, have shown promise as an alternative to in-feed antibiotics in reducing enteric diseases and eliminating subsequent contamination of poultry products. Although the manner by which DFM act remains to be clarified, they are thought to function by maintaining the presence of beneficial commensal microorganisms in the gut thereby providing an optimally balanced population among the diverse microfloral species (Reid and Friendship, 2002; Patterson and Burkholder, 2003; Dahiya et al., 2006; Callaway et al., 2008). Mechanistically, DFM influence the intestinal microbiota in multiple and diverse ways, including competitive exclusion of pathogenic bacteria, regulation of the local mucosal cell-mediated immune responses, increasing antibody production, promoting epithelial barrier integrity, reducing epithelial cell apoptosis, enhancing dendritic cell-induced T cell hyporesponsiveness, improving T cell homing to mesenteric lymph nodes, and augmenting toll-like receptor signaling (Ng et al., 2009).

The most common DFM are live bacteria or yeast used as feed supplements (Patterson and Burkholder, 2003). Bacteria frequently used as DFM in poultry pro-

Received August 24, 2009.
Accepted October 19, 2009.
[1]Corresponding author: Hyun.Lillehoj@ars.usda.gov

duction include species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Escherichia*, *Lactobacillus*, *Lactococcus*, and *Streptococcus*. Among these, nonpathogenic *Bacillus* spp. have been extensively studied and widely employed in many commercial applications (Hong et al., 2005). Spores of *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus clausii* have been used as DFM in animals and humans. Strains of *B. subtilis* have been selected as candidate DFM on the basis of their in vitro inhibitory effect on avian pathogenic *Escherichia coli* or *Clostridium perfringens* (Gebert et al., 2006). Supplementation with a *Bacillus*-based DFM was shown to improve feed conversion in poultry (Gebert et al., 2007) and pigs (Davis et al., 2008) and to beneficially alter the gastrointestinal microflora to reduce colonization by avian pathogenic *E. coli* and *C. perfringens* type A (Gebert et al., 2007). In addition, it has been proposed that *Bacillus*-based DFM enhance immune function and promote the synthesis of endogenous antimicrobial peptides in the gut (Hong et al., 2005). In spite of these reports, limited information exits concerning the mechanisms through which DFM influence host immunity in chickens. Therefore, this study was conducted to investigate the effects of selected DFM on the growth performance and gut morphology in broiler chickens and to correlate these effects with changes in various parameters of intestinal immunity that are associated with protection against infection by enteric pathogens.

MATERIALS AND METHODS

DFM

Eight individual *B. subtilis* strains, designated Bs2084, LSSAO1, 3AP4, Bs18, 15AP4, 22CP1, Bs27, and Bs278, and 1 multiple-strain DFM product, Avicorr (Danisco/Agtech Products Inc., Waukesha, WI) were used. These *Bacillus* strains were isolated from various sources including poultry litter, swine lagoon, rumen fluid, and other agricultural environments. Direct-fed microbials were selected based on their inhibitory effects on avian pathogenic *E. coli* or *C. perfringens* type A (Rehberger and Jordan-Parrott, 2005). Avicorr contains equal amounts of Bs2084, LSSAO1, and 15AP4; is generally recognized as safe by the US Food and Drug Administration; and is approved for feeding to animals by the Association of American Feed Control Officials.

Birds, Diets, and Experimental Design

Two hundred fifty 1-d-old broiler chickens hatched at Longenecker's Hatchery (Elizabethtown, PA) were wing-banded upon arrival, weighed, and randomly placed in Petersime starter brooder units. At 1 and 2 d posthatch, 5.0×10^6 cfu of DFM suspended in 0.5 mL of sterile distilled water were administered to chicks (n = 24/group) by oral gavage. Controls (n = 34) were given carrier alone dissolved in water. All chicks at 1 and 2 d were provided with nonmedicated mash base diets. Beginning at 3 d, chicks were fed ad libitum with nonmedicated mash base diets supplemented with 1.5×10^5 cfu/g of DFM until the end of the experimental period. The control diet was formulated by mixing the base diet with carrier alone. At 15 d posthatch, all chickens were transferred to larger hanging cages. No adverse events on the chicks were observed throughout the 22-d experimental period. Body weights were measured at 7, 14, and 21 d. All experimental protocols were approved by the Small Animal Care Committee of the Beltsville Agricultural Research Center.

Immunization with Recombinant Eimeria Profilin Protein

To evaluate the effect of dietary DFM on an antibody response, 3 birds/group were immunized subcutaneously with 50 μg/mL of the 3–1E recombinant profilin coccidial protein (Lillehoj et al., 2005, 2007) in 0.1 mL of Freund's complete adjuvant at 8 d and boosted with the equivalent amount in Freund's incomplete adjuvant at 15 d (Figure 1).

Collection of Samples

Five birds per group at 7 d and 3 birds/group at 14 and 21 d posthatch were selected for collection of blood, spleen, and small intestine samples. Cervical dislocation was used to euthanatize the birds by the well-trained personnel as proposed by AVMA (2007). Immediately after euthanasia, blood was obtained by cardiac puncture, and the spleen and intestines were immediately removed. In addition, at 22 d, blood was drawn from 3 birds/group that had been immunized with the 3–1E protein. Spleens were processed to measure splenocyte proliferation on the day of euthanasia. A section of tissue from the duodenum to the ileum was used to measure intestinal intraepithelial lymphocyte (**IEL**) subpopulations, cytokine mRNA levels, and intestinal villus-crypt morphometry. For measurement of cytokine mRNA, sections approximately 20 cm long that were anterior and posterior to the diverticulum were taken at 14 d. The remainder of the intestine was used for IEL subpopulation measurements, except for those sampled at 21 d when 1.0 cm was taken for morphometry from the duodenum (midpoint of ascending duodenum), the jejunum (10 cm anterior to the diverticulum), and the ileum (10 cm posterior to the diverticulum).

Villus and Crypt Morphometry

Intestinal samples were fixed in 10% phosphate-buffered formalin for a minimum of 48 h, and 4.0-μm sections were prepared commercially (American Histolab Inc., Gaithersburg, MD). The sections were stained with standard hematoxylin-eosin solution and observed

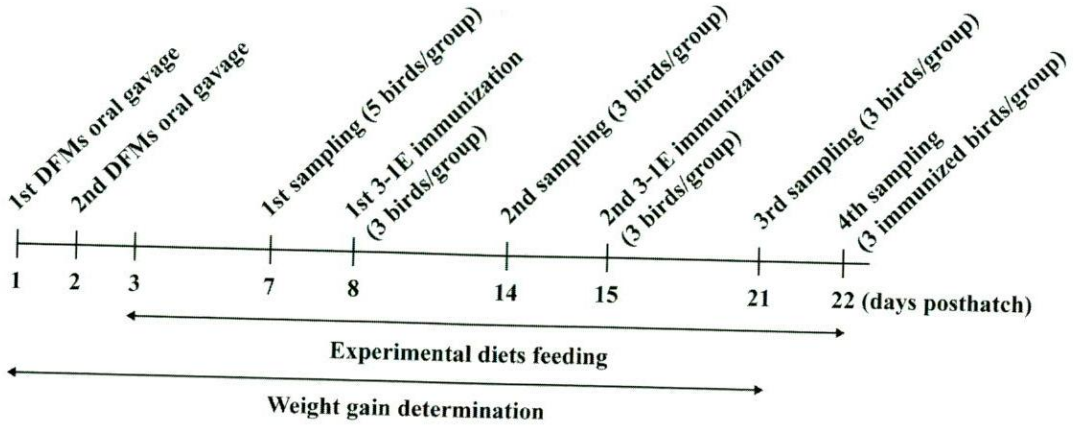

Figure 1. Schematic outline of the experimental design. Chickens were orally gavaged with direct-fed microbials (DFMs) at 1 and 2 d and experimental diets were provided from 3 d posthatch until the end of the experiment. Chickens were immunized with *Eimeria* recombinant profilin protein (3-1E) at 8 and 15 d, and serum samples were collected at 22 d posthatch. Blood, spleen, and intestine samples were obtained at 7, 14, and 21 d posthatch. Body weight gain was assessed between 1 and 21 d posthatch.

for villus height and crypt depth at 100× magnification by light microscopy (CH30, Olympus, Tokyo, Japan) using a calibrated ocular micrometer. Ten microscopic fields per bird were measured.

Spleen Lymphocyte Proliferation

Spleen lymphocyte proliferation in response to medium alone (control), concanavalin A [**ConA**, 5.0 μg/mL; Sigma, St. Louis, MO), or *Salmonella* Typhimurium lipopolysaccharide (**LPS**, 5.0 μg/mL; Sigma) was measured by cellular incorporation of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium as described by Lee et al. (2008). The stimulation index was calculated as the ratio of the mean optical density ($\mathbf{OD_{450}}$) value of mitogen-stimulated cells divided by the OD_{450} value of medium alone-stimulated cells.

Intestinal IEL Subpopulations

Intestinal sections between the duodenum and the ileum were excised, cut longitudinally, and washed with ice-cold Hanks' balanced salt solution without calcium chloride and magnesium sulfate (Sigma). The IEL were isolated by density gradient centrifugation as described (Dalloul et al., 2002).

The IEL obtained were then analyzed using a FACSAria II flow cytometer (BD Biosciences, San Jose, CA). The cells were stained using monoclonal antibodies (**mAb**; USDA-Agricultural Research Service, Beltsville, MD) against the following surface markers: HB2 (human T cells, negative control), K55 (total chicken lymphocytes, positive control), K1 (chicken macrophages-thrombocytes), CD4 (chicken T helper lymphocytes), CD8 (chicken cytotoxic T lymphocytes), TCR1 (chicken γδ T cell receptor), TCR2 (chicken αβ TCR), CD3 (chicken T cells), and BU1 (chicken B cells). Lymphocyte subpopulations were expressed as the percentage of total lymphocytes stained with mAb K55.

Cytokine mRNA Levels in Intestinal IEL

Total RNA extraction, cDNA synthesis, and quantitative reverse transcription-PCR were performed as described (Hong et al., 2006a,b; Lee et al., 2008; Park et al., 2008). Standard curves were generated by using $\log_{10}$-diluted standard RNA, and levels of individual transcripts were normalized to those of glyceraldehyde 3-phosphate dehydrogenase analyzed by the Q-gene program (Hong et al., 2006a,b). The PCR primers for interferon (**IFN**)-α, IFN-γ, interleukin (**IL**)-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17α, tumor necrosis factor superfamily 15 (**TNFSF15**), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, negative control) are listed in Table 1.

Chicken α-1-Acid Glycoprotein ELISA

Chicken α-1-acid glycoprotein (**α-1-AGP**) in serum was measured by ELISA (Life Diagnostics Inc., West Chester, PA) according to the instructions of the manufacturer. The OD_{450} values were determined with an automated microplate reader (Bio-Rad, Richmond, CA).

Measurement of Eimeria Profilin-Specific Antibody Responses

An in-house ELISA was used to measure antibody levels against *Eimeria* profilin (3–1E) in sera collected at 22 d posthatch (7 d after secondary immunization; Lillehoj et al., 2005). Briefly, microtiter plates were coated overnight with 10 μg/well of purified recombinant 3–1E protein, washed with PBS containing 0.05% Tween 20, and blocked with PBS containing 1% BSA. Sera (100 μL/well) were incubated for 1 h at

room temperature with gentle agitation, the wells were washed, and bound antibody was detected with peroxidase-conjugated rabbit anti-chicken IgG (Sigma) and peroxidase-specific substrate. The OD_{450} values were measured with a microplate reader (Bio-Rad).

Statistical Analysis

All data were subjected to 1-way ANOVA using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Mean values of treatment groups were compared using Duncan's multiple range test with $P < 0.05$ considered statistically significant.

RESULTS

Effect of DFM on Growth Performance and Gut Morphometry

Chickens that were fed a diet containing any of the 9 DFM tested in this study did not statistically exhibit altered BW gains between 0 and 21 d posthatch compared with birds fed the nonsupplemented control diet (Table 2). Although not statistically significant, chickens fed a diet containing Bs27 tended to have the greatest BW gains among the treatment groups, followed by the LSSAO1 and 15AP4 groups, respectively. Birds on the 22CP1-supplemented diet had the least weight gain. Chickens fed diets supplemented with 15AP4 or Avicorr had greater duodenal villus height compared with birds given the control diet (Table 2). Villus height in the jejunum was significantly higher in birds fed diets supplemented with 3AP4, Bs18, 15AP4, 22CP1, Bs27, Bs278, or Avicorr compared with nonsupplemented controls. Ileum villus height was increased in birds fed diets supplemented with Bs27, Bs278, or Avicorr versus the control group. Increased crypt depth was observed in the duodenum of birds fed 15AP4, 22CP1, or Bs27; in the jejunum of chickens fed 22CP1 or Bs27; and in the ileum of birds fed with 15AP4 or Bs278 compared with the control group. On the other hand, crypt depth was significantly decreased in the jejunum of birds fed diets containing LSSAO1 as well as in the ileum of those given Bs2084, 3AP4, or Bs18 compared with controls. Ratios of villus height to crypt depth were decreased in the duodenum of birds fed Bs27 or 22CP1 and in the ileum of chickens fed 15AP4 but were increased in the jejunum of birds provided with LSSAO1 or 15AP4 and in the ileum of chickens fed Bs2084, 3AP4, Bs18, or Bs27 compared with controls.

Effect of DFM on Serum α-1-AGP Levels

In general, serum α-1-AGP concentrations increased between 7 and 21 d posthatch in the control and treatment groups (Table 3). However, the increase in α-1-AGP levels was attenuated in the DFM-fed groups compared with the control group. At 7 d posthatch, serum α-1-AGP levels were equal in the control and DFM birds. At 14 d, α-1-AGP levels were lower in chickens

Table 1. Oligonucleotide primers used for quantitative reverse transcription-PCR of chicken cytokines

Type[1]	RNA target[2]	Primer sequence[3]	PCR product size (bp)	GenBank accession no.
Reference	GAPDH	F: 5′-GGTGGTGCTAAGCGTGTTAT-3′ R: 5′-ACCTCTGTCATCTCTCCACA-3′	264	K01458
Proinflammatory	IFN-α	F: 5′-GACATCCTTCAGCATCTCTTCA-3′ R: 5′-AGGCGCTGTAATCGTTGTCT-3′	238	AB021154
	IL-1β	F: 5′-TGGGCATCAAGGGCTACA-3′ R: 5′-TCGGGTTGGTTGGTGATG-3′	244	Y15006
	IL-6	F: 5′-CAAGGTGACGGAGGAGGAC-3′ R: 5′-TGGCGAGGAGGGATTTCT-3′	254	AJ309540
	IL-17α	F: 5′-CTCCGATCCCTTATTCTCCTC-3′ R: 5′-AAGCGGTTGTGGTCCTCAT-3′	292	AJ493595
	TNFSF15	F: 5′-CCTGAGTATTCCAGCAACGCA-3′ R: 5′-ATCCACCAGCTTGATGTCACTAAC-3′	292	NM010245578
Th-1	IFN-γ	F: 5′-AGCTGACGGTGGACCTATTATT-3′ R: 5′-GGCTTTGCGCTGGATTC-3′	259	Y07922
	IL-2	F: 5′-TCTGGGACCACTGTATGCTCT-3′ R: 5′-ACACCAGTGGGAAACAGTATCA-3′	256	AF000631
	IL-12	F: 5′-AGACTCCAATGGGCAAATGA-3′ R: 5′-CTCTTCGGCAAATGGACAGT-3′	274	NM213571
Th-2	IL-4	F: 5′-ACCCAGGGCATCCAGAAG-3′ R: 5′-CAGTGCCGGCAAGAAGTT-3′	258	AJ621735
	IL-10	F: 5′-CGGGAGCTGAGGGTGAA-3′ R: 5′-GTGAAGAAGCGGTGACAGC-3′	272	AJ621614
	IL-13	F: 5′-CCAGGGCATCCAGAAGC-3′ R: 5′-CAGTGCCGGCAAGAAGTT-3′	256	AJ621735

[1]Th = T helper cell.
[2]GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IFN = interferon; IL = interleukin; TNFSF15 = tumor necrosis factor superfamily 15.
[3]F = forward primer; R = reverse primer.

Table 2. Effects of direct-fed microbials on BW gains and villus morphology

Measure	Control	Bs2084	LSSAO1	3AP4	Bs18	15AP4	22CP1	Bs27	Bs278	Avicorr[1]	Pooled SEM	*P*-value
Weight gain/bird[2] (g)	760.1	729.0	792.6	766.8	729.2	784.9	693.1	842.0	755.7	775.8	39.4	0.091
Villus height[3] (μm)												
Duodenum	1,928	1,791	1,828	1,999	1,948	2,127*	1,936	2,045	1,799	2,138*	6.6	<0.001
Jejunum	821	778	924	1,113*	996*	1,212*	1,045*	1,120*	999*	1,080*	4.3	<0.001
Ileum	760	713	716	745	807	798	809	948*	1,011*	931*	2.9	<0.001
Crypt depth[3] (μm)												
Duodenum	177	180	177	178	207	229*	272*	250*	173	200	1.9	<0.001
Jejunum	151	131	109†	158	163	173	186*	184*	154	168	0.9	<0.001
Ileum	169	119†	179	130†	122†	240*	175	159	208*	173	1.0	<0.001
Villus:crypt ratio[3]												
Duodenum	11.2	10.3	10.9	11.6	9.9	9.5	7.3†	8.5†	10.8	11.4	0.7	<0.001
Jejunum	5.7	6.1	8.5*	7.1	6.2	7.2*	5.8	6.2	6.9	6.5	0.5	<0.003
Ileum	4.6	6.2*	4.5	5.8*	6.9*	3.5†	4.7	6.1*	5.1	5.5	0.4	<0.001

[1]Danisco/Agtech Products Inc., Waukesha, WI.

[2]Body weight gains were measured between 0 and 21 d posthatch.

[3]Each value is the mean of 10 microscopic fields/bird with 3 birds/treatment (n = 30).

*Significantly increased height-depth compared with the control group ($P < 0.05$).

†Significantly decreased height-depth compared with the control group ($P < 0.05$).

fed Bs2084, LSSAO1, 3AP4, 15AP4, 22CP1, Bs27, or Bs278 but were higher in chickens fed Bs18 or Avicorr compared with controls. At 21 d, α-1-AGP levels were lower in chickens fed all of the DFM diets compared with the control group.

Effect of DFM on Serum Antibody Response to Eimeria Profilin Protein

The effect of DFM on humoral immunity was assessed by measuring serum antibody responses after immunization and boosting with an *Eimeria* profilin recombinant protein. As shown in Figure 2, only birds fed 15AP4 showed elevated profilin-specific antibody levels compared with the unmodified control diet.

Effect of DFM on Spleen Lymphocyte Proliferation

At 7 d posthatch, splenocyte proliferation induced by the T cell mitogen ConA was greater in chickens fed 15AP4, 22CP1, or Avicorr compared with controls and was greater after stimulation with the B cell mitogen LPS in the Bs2084, LSSAO1, 3AP4, Bs18, and Avicorr groups compared with nonsupplemented controls (Figure 3). On the contrary, significantly low LPS-induced splenocyte proliferation was seen in birds fed Bs27 compared with the nonsupplemented control group. At 14 d, spleen cell proliferation in response to ConA was decreased in birds given Bs2084 or LSSAO1 diets but increased after ConA stimulation in birds fed Avicorr and after LPS stimulation in birds fed Bs278

Table 3. Effects of direct-fed microbials on serum α-1-acid glycoprotein (α-1-AGP) levels

	α-1-AGP[1] (μg/mL)		
Treatment	7 d posthatch	14 d posthatch	21 d posthatch
Control	95	241	791
Bs2084	121	170†	292†
LSSAO1	94	92†	168†
3AP4	78	141†	356†
Bs18	100	338*	286†
15AP4	92	135†	112†
22CP1	61	130†	206†
Bs27	125	108†	209†
Bs278	99	118†	105†
Avicorr[2]	123	410*	138†
Pooled SEM	5.7	9.4	14.4
P-value	<0.001	<0.001	<0.001

[1]α-1-AGP levels were measured with 5 (7 d) or 3 (14 and 21 d) birds per treatment group.

[2]Danisco/Agtech Products Inc., Waukesha, WI.

*Significantly increased α-1-AGP compared with the control group.

†Significantly decreased α-1-AGP compared with the control group.

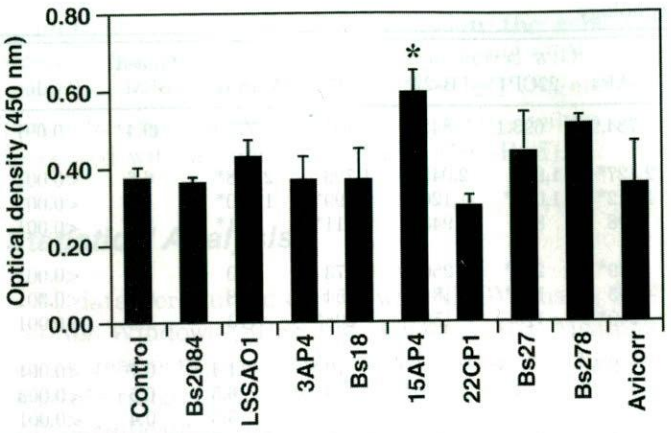

Figure 2. Antibody response in chickens immunized with *Eimeria* recombinant profilin protein (3-1E) and fed diets with or without direct-fed microbials. Three chickens per treatment group were immunized with 3-1E at 8 and 15 d, and serum samples were collected 7 d after the second immunization. Each bar represents the mean ± SD of triplicate samples. The asterisk (*) denotes significantly increased antibody levels compared with the control group ($P < 0.05$). Avicorr was provided by Danisco/Agtech Products Inc. (Waukesha, WI).

or Avicorr. At 21 d, ConA-stimulated proliferation was increased in the 15AP4, Bs27, and Bs278 groups and decreased in the LSSAO1, Bs18, and 22CP1 groups, whereas LPS-stimulated proliferation was increased in the LSSAO1 group and decreased in the 3AP4, 22CP1, and Bs27 groups.

Effect of DFM on Intestinal IEL Subpopulations

At 7 d posthatch, $TCR2^+$ IEL were undetectable in chickens fed the nonsupplemented diet but were detected in all of the DFM-fed groups, ranging from 5.1 to 17.7% of total IEL (Table 4). The $K1^+$ and $BU1^+$ IEL were relatively low. At this time, $CD3^+$, $CD4^+$, $CD8^+$, and $TCR1^+$ subpopulations also were increased in 3 or 4 of the 9 groups on DFM diets compared with the control group. Of note, chickens fed the Avicorr-supplemented diet exhibited 84.8% $CD8^+$ cells compared with 14.8% in the control group, a 5.7-fold increase. At 14 d posthatch, $CD8^+$ IEL remained greater than 50% of the total lymphocyte population in 7 of the 9 experimental groups (Bs2084, LSSAO1, Bs18, 15AP4, Bs27, Bs278, and Avicorr; Table 5). With the exception of the macrophage-thrombocyte $K1^+$ subpopulation, the majority of changes observed in the $CD4^+$, $TCR1^+$, $TCR2^+$, and $BU1^+$ cells of DFM-fed chickens were decreased compared with birds given the control diet. The percentage of $CD3^+$ IEL was high in Bs27 and Bs278 groups but low in LSSAO1, Bs18, and 22CP1 groups compared with the control group. At 21 d posthatch, all subpopulation changes that were induced by the supplemented diets were increased compared with controls and none of the subpopulations examined were decreased (Table 6). The 30.4-fold increase in $CD4^+$ cells from 0.7% in the control group to 21.3% in the Bs278 group represented the greatest relative increase observed among all treatment groups at all time points examined. As an additional negative control, the percentage of cells stained by the human T cell-specific HB2 mAb was consistently less than 2% in all treatment groups (data not shown).

Effect of DFM on Cytokine mRNA Levels in IEL

The levels of mRNA encoding proinflammatory (IFN-α, IL-1β, IL-6, IL-17α, TNFSF15), T helper (**Th**)-1 type (IFN-γ, IL-2, IL-12), and Th-2 type (IL-4, IL-10, IL-13) cytokines were quantified by real-time reverse transcription-PCR in IEL at 14 d posthatch from chickens fed control or DFM-supplemented diets. For the proinflammatory cytokines (Figure 4), IFN-α transcripts were increased by Bs2084, LSSAO1, 3AP4, and 22CP1 and were decreased by 15AP4 and Bs27. The IL-1β transcripts were increased by LSSAO1 and 3AP4 and were decreased by Bs27. The IL-6 transcripts were increased by LSSAO1, 22CP1, and Avicorr. The IL-17α transcripts were increased by Avicorr, and TNFSF15 transcripts were increased by 3AP4, Bs278, and Avicorr. In particular, the 294-fold increase in IL-17α transcripts in IEL from chickens fed Avicorr represented the greatest increase observed among all cytokines in all treatment groups. For the Th-1-type cytokines (Figure 5), IFN-γ mRNA were increased by 22CP1 and were decreased by all remaining DFM. The IL-2 mRNA were increased by 22CP1 and were decreased by Bs18, 15AP4, and Bs27. The IL-12 mRNA were increased by Bs278 and Avicorr. For the Th-2-type cytokines (Figure 6), IL-4 transcripts were increased by LSSAO1, Bs278, and Avicorr; IL-10 transcripts were increased by LSSAO1, Bs18, and 22CP1; and IL-13 transcripts were increased by LSSAO1, 22CP1, and Avicorr. None of the Th-2 cytokines analyzed were decreased by any of the DFM diets.

DISCUSSION

This study was conducted to investigate the effects of 9 selected DFM on broiler chicken growth performance, gut villus and crypt morphometry, and local and systemic inflammation-immunity. Our results demonstrated that although BW gains were unaffected by any of the DFM, chickens that were fed 7 of the 9 experimental diets displayed increased villus height or crypt depth, or both, compared with control diet-fed birds. In addition, serum concentrations of the α-1-AGP acute phase protein as an index of nonspecific inflammation were generally depressed in DFM-fed birds compared with birds on the control diet, especially at 14 and 21 d posthatch. although antibody responses after immunization with *Eimeria* profilin protein as a measure of humoral immunity were, by in large, unaffected by DFM diets, spleen cell proliferative responses to T and B cell

mitogens were increased or decreased compared with control diet birds. However, no consistent patterns of altered mitogen-induced proliferation were evident at 7, 14, and 21 d posthatch with any particular DFM. At 7 d posthatch, chickens fed diets containing 15AP4, Bs27, or Avicorr had a higher percentage of intestinal IEL expressing T cell surface markers (CD3, CD4, CD8, TCR1, TCR2), whereas most DFM decreased the percentage of IEL expressing the BU1 B cell marker at this time. Interestingly, cells expressing the K1 macrophage-thrombocyte marker were increased by Bs18, 15AP4, 22CP1, Bs27, and Avicorr at 14 d posthatch but were unaffected at 7 and 21 d. Finally, dietary DFM generally increased the levels of IEL transcripts for proinflammatory, Th-1-type, and Th-2-type cytokines, with the notable exception of IFN-γ, which was reduced by

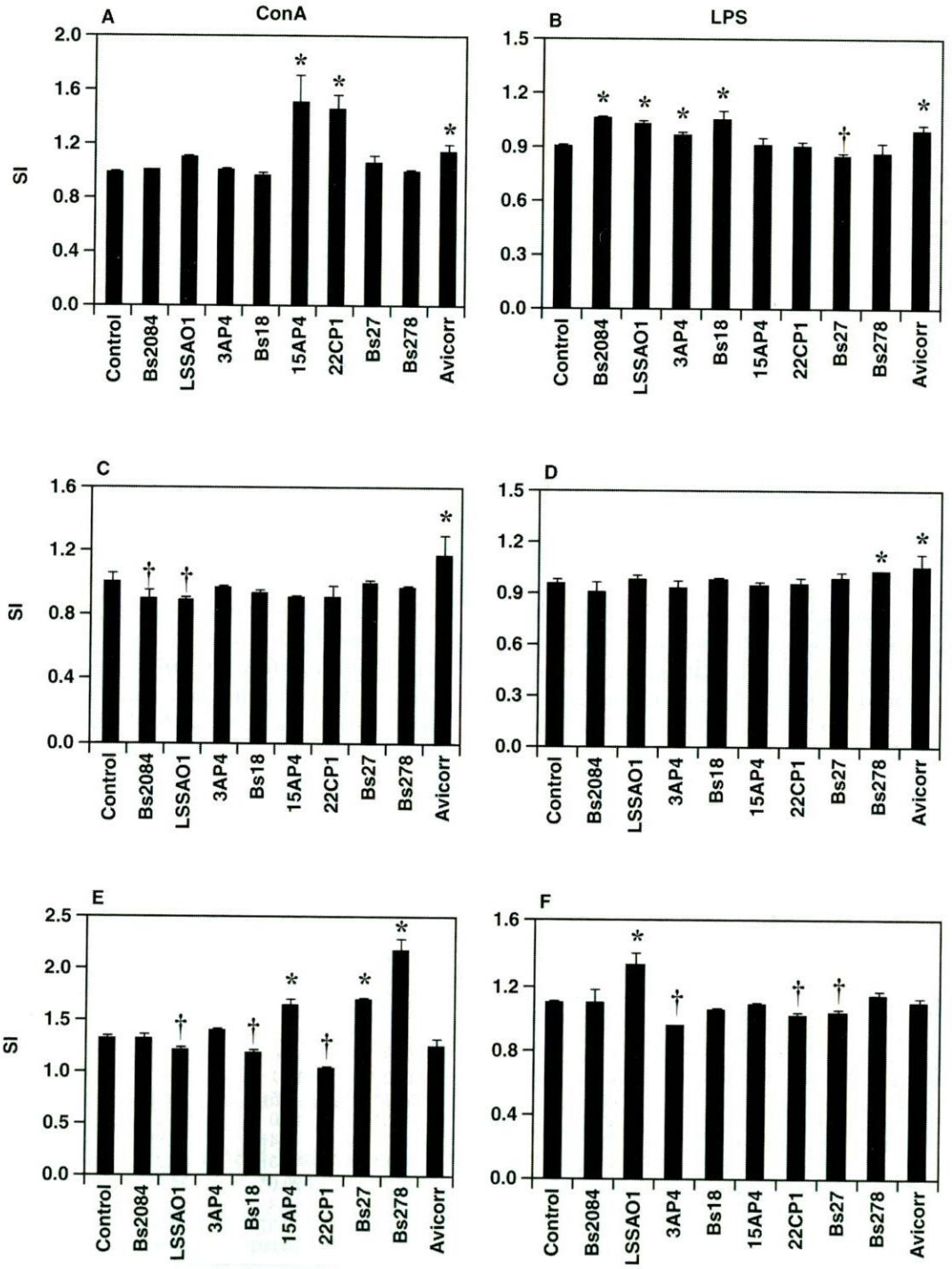

Figure 3. Effect of dietary direct-fed microbials on spleen lymphocyte proliferation. Spleen lymphocytes obtained at 7 d (A, B), 14 d (C, D), or 21 d (E, F) posthatch were stimulated with concanavalin A (ConA) or lipopolysaccharide (LPS), and the stimulation index (SI) for each treatment was calculated as described in the Materials and Methods. Each bar represents the mean ± SD of triplicate samples. The asterisk (*) denotes significantly increased proliferation compared with the control group and the dagger (†) denotes significantly decreased proliferation compared with the control group ($P < 0.05$). Avicorr was provided by Danisco/Agtech Products Inc. (Waukesha, WI).

Table 4. Effects of direct-fed microbials on intestinal intraepithelial lymphocyte subpopulations at 7 d posthatch

	Cell surface marker[1]						
Treatment	K1	CD3	CD4	CD8	TCR1	TCR2	BU1
Control	0.3	15.7	0.9	14.8	6.6	0.0	2.7
Bs2084	0.6	17.5	1.1	15.8	4.3†	13.0*	2.0†
LSSAO1	0.3	17.1	0.5	29.9*	7.3	5.6*	2.2†
3AP4	0.0	10.2†	0.0†	11.5	2.3†	7.2*	1.0†
Bs18	0.0	7.9†	0.0†	12.5	10.2*	5.6*	1.5†
15AP4	1.7	31.6*	4.3*	28.4*	12.3*	4.7*	4.1*
22CP1	0.0	14.9	0.0†	20.1	3.6†	7.3*	2.6
Bs27	0.0	37.3*	5.0*	39.8*	13.7*	17.7*	0.0†
Bs278	1.9	7.9†	1.4*	14.2	4.2†	5.1*	0.0†
Avicorr[2]	0.6	24.5*	1.5*	84.8*	10.1*	15.7*	1.7†
Pooled SEM	0.03	0.654	0.070	1.088	0.264	0.307	0.068
P-value	NE[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Each value represents the mean percentage of cells expressing the indicated surface marker compared with cells expressing the K55 pan lymphocyte marker from 5 birds/treatment group measured at 7 d posthatch.

[2]Danisco/Agtech Products Inc., Waukesha, WI.

[3]NE = nonexpressed statistical analysis due to the low observation levels.

*Significantly increased percentage of cells compared with the control group ($P < 0.05$).

†Significantly decreased percentage of cells compared with the control group ($P < 0.05$).

all DFM except 22CP1. Taken together, these data are consistent with the hypothesis that the DFM used in this study modulate a diverse set of physiologic- and immune-related parameters in broiler chickens.

Prior studies on the effects of dietary DFM on broiler growth performance have been controversial. Although some reports have shown their effects to be beneficial (Zhang et al., 2005a,b; Nayebpor et al., 2007; Talebi et al., 2008), others have found no or minimal effect (Lee et al., 2005; Mountzouris et al., 2007; Willis et al., 2007; Willis and Reid, 2008). Our results tend to support the latter group of studies, although the increase in BW gain produced by the Bs27-containing diet (842 g vs. 760 g for the control) may require further dose-response and time course analyses to achieve statistical significance.

Because the modulation of gut microflora by dietary DFM in chickens is well-documented (Hariharan et al., 2004; Nava et al., 2005; Yang et al., 2009), the DFM used in this study were selected under the presumption that they act by decreasing the intestinal load of harmful bacteria, thereby promoting gut function, increasing immunity-mediated pathogen protection and nutrient absorption, and enhancing weight gain. In this regard, future studies using broilers fed with less digestible diets, reared under field conditions or exposed to common enteric infections, or both, may delineate a positive effect of dietary DFM on growth performance.

Given the paucity of published literature reports documenting the effects of DFM on immune responses in naïve broilers, the main focus of the current investigation was to ascertain the manner by which these

Table 5. Effects of direct-fed microbials on intestinal intraepithelial lymphocyte subpopulations at 14 d posthatch

	Cell surface marker[1]						
Treatment	K1	CD3	CD4	CD8	TCR1	TCR2	BU1
Control	1.4	34.2	10.5	33.9	12.3	36.4	5.0
Bs2084	1.9	28.5	9.2	58.5*	14.7	33.6	6.3
LSSAO1	1.7	17.5†	9.4	51.8*	10.0	27.2†	1.8†
3AP4	1.7	27.5	9.5	43.2	13.1	32.8	6.3
Bs18	2.4*	17.6†	5.8†	54.1*	8.5†	14.7†	3.6†
15AP4	2.8*	32.4	7.5†	67.3*	12.0	34.1	2.6†
22CP1	4.6*	8.8†	5.1†	35.5	5.4†	13.8†	4.2
Bs27	2.3*	46.8*	5.4†	59.6*	8.5†	31.2	2.3†
Bs278	1.0	44.9*	11.4	67.2*	20.0*	24.9†	2.9†
Avicorr[2]	5.3*	28.2	17.4*	50.1*	9.3	27.2†	6.3
Pooled SEM	0.090	0.974	0.308	1.686	0.379	0.904	0.141
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Each value represents the mean percentage of cells expressing the indicated surface marker compared with cells expressing the K55 pan lymphocyte marker from 5 birds/treatment group measured at 14 d posthatch.

[2]Danisco/Agtech Products Inc., Waukesha, WI.

*Significantly increased percentage of cells compared with the control group ($P < 0.05$).

†Significantly decreased percentage of cells compared with the control group ($P < 0.05$).

Table 6. Effects of direct-fed microbials on intestinal intraepithelial lymphocyte subpopulations at 21 d posthatch

	Cell surface marker[1]						
Treatment	K1	CD3	CD4	CD8	TCR1	TCR2	BU1
Control	0.2	12.8	0.7	13.7	10.0	8.0	8.0
Bs2084	0.0	15.6	4.1*	10.0	9.1	5.0	7.1
LSSAO1	0.0	33.6*	2.9	18.2	19.3*	27.1*	29.6*
3AP4	0.0	50.9*	5.1*	21.3	36.4*	16.9*	8.4
Bs18	0.6	41.6*	12.4*	44.7*	23.8*	18.6*	12.0
15AP4	0.0	35.4*	0.9	22.6	22.4*	10.5	16.3*
22CP1	0.6	43.1*	2.1	25.8*	25.8*	30.8*	28.4*
Bs27	0.4	47.9*	12.9*	56.8*	23.7*	46.0*	26.5*
Bs278	0.0	71.4*	21.3*	60.5*	14.2	27.0*	22.9*
Avicorr[2]	0.4	46.9*	6.6*	33.1*	20.4*	34.9*	9.4
Pooled SEM	0.01	1.362	0.296	1.106	0.692	0.811	0.600
P-value	NE[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Each value represents the mean percentage of cells expressing the indicated surface marker compared with cells expressing the K55 pan lymphocyte marker from 5 birds/treatment group measured at 14 d posthatch.

[2]Danisco/Agtech Products Inc., Waukesha, WI.

[3]NE = nonexpressed statistical analysis due to the low observation levels.

*Significantly increased percentage of cells compared with the control group ($P < 0.05$).

experimental diets modulated various parameters of inflammation, humoral immunity, and cellular immunity. It has been found that α-1-AGP is an acute phase protein that contributes to restoring homeostasis and restricting microbial growth in an antibody-independent manner after infection, inflammation, or stress (Murata et al., 2004). In addition, α-1-AGP is known to possess antiinflammatory and antibacterial properties (Hochepied et al., 2003). The most common acute phase proteins in avians are α-1-AGP, ceruloplasmin, serum amyloid-A, and transferrin that may increase up to 100-fold after inflammatory stimulation (Takahashi et al., 1998; Kefali and Toker, 2006; Juul-Madsen et al., 2008). In the present study, dietary DFM generally reduced α-1-AGP levels, reflecting a lower inflammatory status compared with the control group. We hypothesize that this effect is secondary to reduction of inflammatory signals in the gut as a consequence of the exclusion of potentially harmful microbiota by DFM (Dalloul et al., 2003; Gebert et al., 2007; Lee et al., 2007a,b; Higgins et al., 2008; Vila et al., 2009). In spite of these results, others have shown no effect on plasma ceruloplasmin or increased AAP levels after feeding DFM to broiler chickens (Kefali and Toker, 2006; Lange, 2007). Possible differences in bacterial species, doses, feeding schedules, and animal age may underlie some of these discrepancies as well as the overriding conditions of low stress, low subacute challenge presented by the caged study.

The antibody response of birds that had been immunized with the coccidial 3–1E protein while receiving a 15AP4-supplemented diet was significantly enhanced compared with the control group. Increased anti-3–1E antibody levels also were induced by feeding Bs27 or Bs278, but did not reach statistical significance. In line with these results, antibody titers to SRBC as a model antigen, as well as to a Newcastle disease virus vaccine, were elevated in birds fed with DFM-supplemented diets compared with nonsupplemented diets (Haghighi et al., 2005; Khaksefidi and Ghoorchi, 2006; Li et al., 2009). Increased humoral immunity in DFM-fed chickens has been attributed to increased proliferative and functional activities of antibody-producing B cells (Panda et al., 2008). In this study, we used Freund's complete or incomplete adjuvant for primary and secondary immunization with the coccidial 3–1E protein. Although Freund's adjuvant has been used in recombinant *Eimeria* vaccine (Subramanian et al., 2008), subtle effects derived from the DFM might be masked because Freund may induce strong immune response. In this regard, a less active adjuvant such as aluminum hydroxide (Asif et al., 2004) would be more appropriate to see, if any, the immune adjuvant role of DFM in poultry.

Cell proliferation is an important indicator of lymphocyte function in DFM-fed chickens (Erickson and Hubbard, 2000; Brisbin et al., 2008a). Dietary DFM exhibited strain-specific effects on both B and T cell mitosis (Kirjavainen et al., 1999), whereas other investigators reported no effect of DFM on proliferation (Koenen et al., 2004; Roller et al., 2004). According to in vitro studies, DFM components stimulated splenocyte proliferation (Amrouche et al., 2006) or altered gene expression profiles (Brisbin et al., 2008b). In the present report, single DFM strains as well as the Avicorr 3 component mixture affected both T and B cell responses. The underlying mechanism by which dietary DFM influence splenocyte proliferation has been postulated to involve "cross-talk" between the bacterial cells and the host immune system (Koenen et al., 2004; Corthesy et al., 2007).

Intestinal IEL subpopulations expressing macrophage, T cell, or B cell surface markers were measured on a weekly basis to assess whether dietary DFM affected development of the local immune system in broiler chicken. Intraepithelial lymphocytes are chosen because they constitute the primary immune effector cells in the gut

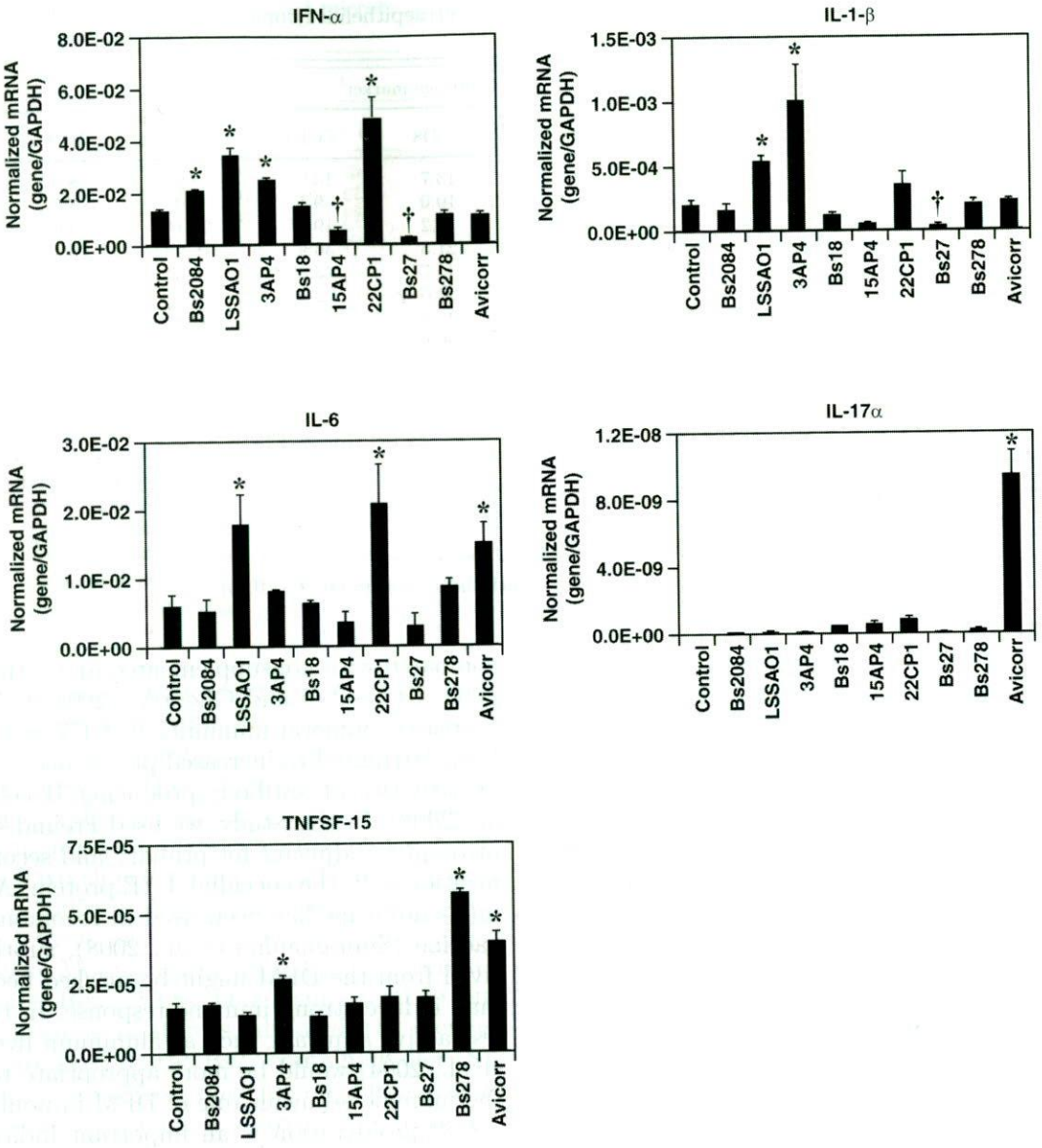

Figure 4. Effects of dietary direct-fed microbials (DFM) on proinflammatory cytokine mRNA levels. Chickens were fed diets with or without DFM, intestinal intraepithelial lymphocytes were isolated at 14 d posthatch, and transcripts for the indicated cytokines were quantified by real-time reverse transcription-PCR and normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts. Each bar represents mean ± SD of triplicate samples. The asterisk (*) denotes significantly increased mRNA levels compared with the control group and the dagger (†) denotes significantly decreased mRNA levels compared with the control group ($P < 0.05$). Avicorr was provided by Danisco/Agtech Products Inc. (Waukesha, WI). IFN-α = interferon-α; IL = interleukin; TNFSF-15 = tumor necrosis factor superfamily 15.

and play a critical role in eliciting protective immunity to enteric pathogens (Lillehoj et al., 2004). Among the DFM examined, 15AP4, Bs27, Bs278, and Avicorr were most effective in enhancing the percentage of IEL T cell subpopulations, particularly $CD8^+$ cells. These results complement our earlier report demonstrating increased $CD3^+$, $CD4^+$, $CD8^+$, and $TCR2^+$ IEL subpopulations in broiler chickens fed a *Lactobacillus*-supplemented diet compared with birds given a DFM-free diet (Dalloul et al., 2003). Similarly, Novak et al. (2007) reported that early application of *Lactobacillus brevis* to turkey poults had the potential to enhance immune development in both the intestine and peripheral blood mononuclear cells. Although IEL percentages are changed by DFM compared with controls, these changes were unlikely to reflect alterations in total IEL numbers based upon the results of our previous probiotic studies (Dalloul et al., 2003; Lee et al., 2007a,b). Stimulation of specific IEL subsets by DFM likely contributes to increased host resistance to enteric pathogens that would otherwise cause clinical disease (Lillehoj and Trout, 1996). Indeed, Lee et al. (2007a,b) demonstrated such an effect in the context of experimental avian coccidiosis using *Pediococcus*- or *Saccharomyces*-based DFM. Given that young chicks are particularly susceptible to infection by opportunistic pathogens due to their immature immune system (Lowenthal et al., 1994; Koenen et al., 2002), application of dietary DFM at an early age would appear to provide the optimal effect on enhancing immune competence.

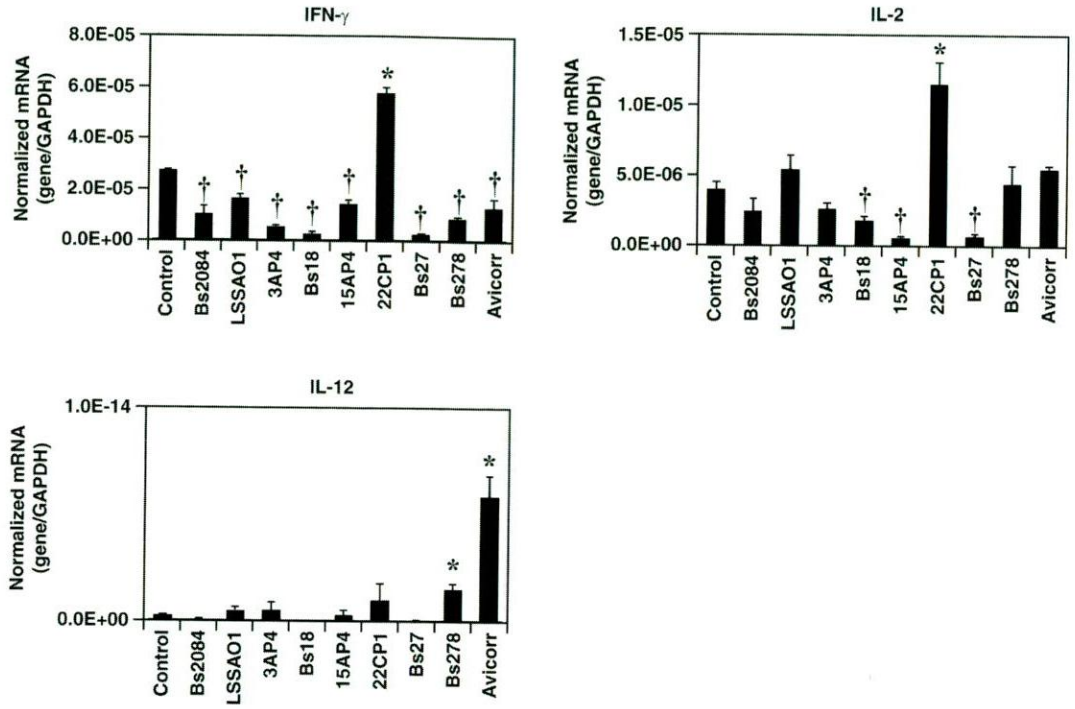

Figure 5. Effects of dietary direct-fed microbials (DFM) on T helper 1-type cytokine mRNA levels. Chickens were fed diets with or without DFM, intestinal intraepithelial lymphocytes were isolated at 14 d posthatch, and transcripts for the indicated cytokines were quantified by real-time reverse transcription-PCR and normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts. Each bar represents mean ± SD of triplicate samples. The asterisk (*) denotes significantly increased mRNA levels compared with the control group and the dagger (†) denotes significantly decreased mRNA levels compared with the control group ($P < 0.05$). Avicorr was provided by Danisco/Agtech Products Inc. (Waukesha, WI). IFN-γ = interferon-γ; IL = interleukin.

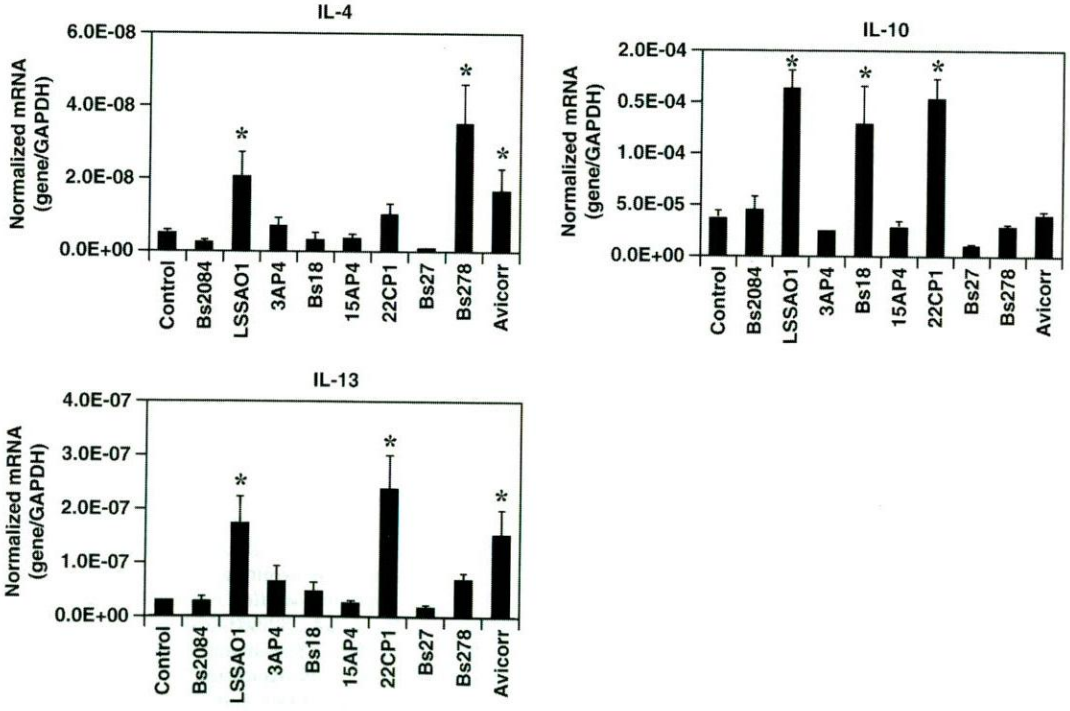

Figure 6. Effects of dietary direct-fed microbials (DFM) on T helper 2-type cytokine mRNA levels. Chickens were fed diets with or without DFM, intestinal intraepithelial lymphocytes were isolated at 14 d posthatch, and transcripts for the indicated cytokines were quantified by real-time reverse transcription-PCR and normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts. Each bar represents mean ± SD of triplicate samples. The asterisk (*) denotes significantly increased mRNA levels compared with the control group ($P < 0.05$). Avicorr was provided by Danisco/Agtech Products Inc. (Waukesha, WI). IL = interleukin.

Based on the fact that IEL subpopulations were altered by DFM feeding, it is not surprising that markedly altered levels of transcripts encoding proinflammatory, Th-1-type, and Th-2-type cytokines also were detected in these cells. Cytokine mRNA levels were increased, decreased, or unchanged compared with controls depending on the DFM used. Chichlowski et al. (2007) reported that chicks fed a diet supplemented with *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium thermophilum*, and *Enterococcus faecium* (PrimaLac, Star Labs Inc., Clarksdale, MO) for 21 d exhibited decreased intestinal mRNA levels of the proinflammatory cytokine IL-6 and increased expression of the antiinflammatory cytokine IL-10. On the other hand, another proinflammatory cytokine, IL-1β, was not affected by the DFM diet. The authors stated, however, that the significance of these alterations was not clear due to the small sample size employed in their study. Repression of IFN-γ and IL-12 expression in the chicken gut was reported to be associated with DFM-mediated reduction in intestinal colonization by *Salmonella enterica* serovar Typhimurium (Haghighi et al., 2008). By contrast, Fujiwara et al. (2009) observed no statistically significant differences in the expression of IFN-γ, IL-3, and IL-4 when birds were fed diets with or without *Bacillus subtilis*-fermented soybean. In a mouse study by Huang et al. (2008), dietary DFM increased IL-6 and tumor necrosis factor-α (TNF-α) in spleen and mesenteric lymph nodes.

Cytokines are immunoregulatory peptides with relatively small molecular weights that participate in innate and adaptive immune responses. Interleukin-2 (IL-2) is a Th-1-associated cytokine that plays a central role in adaptive immunity. Interleukin-12 (IL-12) is an important cytokine required for the initiation and regulation of cellular immunity through the differentiation of naïve T cells into Th-1 cells, which is crucial for host resistance to many microbial pathogens (Park et al., 2008). Interferon-γ (IFN-γ) regulates acquired immunity by activating lymphocytes and enhancing the expression of MHC class II antigens. In addition, IFN-γ is a common marker of cellular immunity and high levels have been correlated with protective immune responses to coccidial infections (Lee et al., 2008). Members of the tumor necrosis factor superfamily play crucial roles in both innate and adaptive immunity, including inflammation, apoptosis, and cell proliferation (Kaiser and Stäheli, 2008). Interleukin-17α (IL-17α) has been implicated in host defense against bacteria, parasites, and viruses including *Mycoplasma pneumoniae*, *Toxoplasma gondii*, human immunodeficiency virus, and *Eimeria* (Min and Lillehoj, 2002). Therefore, because *Bacillus*-based DFM affected the expression of numerous IEL cytokines, it can be expected that they would also influence a diverse array of immune functions.

In conclusion, 8 *Bacillus* spp. and 1 commercial DFM preparation were tested as in-feed supplements for their effect on BW gain, intestinal villus-crypt length-depth, and various parameters of innate and adaptive immunity. Although growth performance was not statistically significant among the different dietary groups and most failed to affect serum antibody levels after immunization with a recombinant *Eimeria* protein, altered intestinal morphometric measurements were readily apparent in DFM-fed chickens compared with controls. In addition, levels of serum acute phase protein α-1-AGP were reduced in DFM-fed birds, whereas mitogen-induced splenic lymphocyte proliferation, intestine IEL subpopulations, and cytokine mRNA levels in IEL were increased, decreased, or unchanged compared with controls depending on the DFM used. Based on these results, further studies are needed to determine whether *Bacillus*-based DFM can augment protective immunity against enteric pathogens in chickens and, if so, whether this protection is induced by alterations in particular intestinal lymphocyte subpopulations or cytokine expression profiles, or both.

ACKNOWLEDGMENTS

This project was supported by a trust agreement established between Agricultural Research Service-USDA and Danisco and partially by the Agricultural Research Service in-house project 1265-32000-086-00D. We thank Marjorie Nichols and Stacy Torreyson (Animal Parasitic Diseases Laboratory) for their technical assistance. We also acknowledge J. P. Dubey in the Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service-USDA, for his help on morphological examination. K. W. Lee is the recipient of a Korean Government Short-Term Overseas Research Fellowship, Ministry of Public Administration and Security, South Korea.

REFERENCES

Amrouche, T., Y. Boutin, G. Prioult, and I. Fliss. 2006. Effects of bifidobacterial cytoplasm, cell wall and exopolysaccharide on mouse lymphocyte proliferation and cytokine production. Int. Dairy J. 16:70–80.

Asif, M., K. A. Jenkins, L. S. Hilton, W. G. Kimpton, A. G. D. Bean, and J. W. Lowenthal. 2004. Cytokines as adjuvants for avian vaccines. Immunol. Cell Biol. 82:638–643.

AVMA. 2007. AVMA guidelines on euthanasia (formerly report of the AVMA panel on euthanasia). http://www.avma.org/issues/animal_welfare/euthanasia.pdf Accessed October 2009.

Brisbin, J. T., J. Gong, and S. Sharif. 2008a. Interactions between commensal bacteria and the gut-associated immune system of the chicken. Anim. Health Res. Rev. 9:101–110.

Brisbin, J. T., H. Zhou, J. Gong, P. Sabour, M. R. Akbari, H. R. Haghighi, H. Yu, A. Clarke, A. J. Sarson, and S. Sharif. 2008b. Gene expression profiling of chicken lymphoid cells after treatment with *Lactobacillus acidophilus* cellular components. Dev. Comp. Immunol. 32:563–574.

Callaway, T. R., T. S. Edrington, R. C. Anderson, R. B. Harvey, K. J. Genovese, C. N. Kennedy, D. W. Venn, and D. J. Nisbet. 2008. Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. Anim. Health Res. Rev. 9:217–225.

Chichlowski, M., J. Croom, B. W. McBride, L. Daniel, G. Davis, and M. D. Koci. 2007. Direct-fed microbial PrimaLac and salinomycin modulate whole-body and intestinal oxygen consumption and intestinal mucosal cytokine production in the broiler chick. Poult. Sci. 86:1100–1106.

Corthesy, B., H. R. Gaskins, and A. Mercenier. 2007. Cross-talk between probiotic bacteria and the host immune system. J. Nutr. 137:781S–790S.

Dahiya, J. P., D. C. Wilkie, A. G. van Kessel, and M. D. Drew. 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Anim. Feed Sci. Technol. 129:60–88.

Dalloul, R. A., H. S. Lillehoj, T. A. Shellem, and J. A. Doerr. 2002. Effect of vitamin A deficiency on host intestinal immune response to *Eimeria acervulina* in broiler chickens. Poult. Sci. 81:1509–1515.

Dalloul, R. A., H. S. Lillehoj, T. A. Shellem, and J. A. Doerr. 2003. Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. Poult. Sci. 82:62–66.

Davis, M. E., T. Parrott, D. C. Brown, B. Z. de Rodas, Z. B. Johnson, C. V. Maxwell, and T. Rehberger. 2008. Effect of a *Bacillus*-based direct-fed microbial feed supplement on growth performance and pen cleaning characteristics of growing-finishing pigs. J. Anim. Sci. 86:1459–1467.

Dekich, M. A. 1998. Broiler industry strategies for control of respiratory and enteric diseases. Poult. Sci. 77:1176–1180.

Erickson, K. L., and N. E. Hubbard. 2000. Probiotic immunomodulation in health and disease. J. Nutr. 130:403S–409S.

Fujiwara, K., M. Yamazaki, H. Abe, K. Nakashima, Y. Yakabe, M. Otsuka, Y. Ohbayashi, Y. Kato, K. Namai, A. Toyoda, Y. Miyaguchi, and Y. Nakamura. 2009. Effect of *Bacillus subtilis* var. *natto* fermented soybean on growth performance, microbial activity in the caeca and cytokine gene expression of domestic meat type chickens. Jpn. Poult. Sci. 46:116–122.

Gebert, S., C. Kromm, and T. Rehberger. 2006. Development of a direct fed microbial to control pathogens associated with turkey poult production. Poult. Sci. 85(Suppl. 1):71. (Abstr.)

Gebert, S., C. Kromm, and T. Rehberger. 2007. Effect of a *Bacillus*-based direct-fed microbial on turkey poult performance and changes within the gastrointestinal microflora. Poult. Sci. 86(Suppl. 1):249. (Abstr.)

Haghighi, H. R., M. F. Abdul-Careem, R. A. Dara, J. Chambers, and S. Shariff. 2008. Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and *Salmonella* infection. Vet. Microbiol. 126:225–233.

Haghighi, H. R., J. Gong, C. L. Gyles, M. A. Hayes, B. Sanei, P. Parvizi, H. Gisavi, J. R. Chambers, and S. Sharif. 2005. Modulation of antibody-medicated immune response by probiotics in chickens. Clin. Diagn. Lab. Immunol. 12:1387–1392.

Hariharan, H., G. A. Murphy, and I. Kempf. 2004. *Campylobacter jejuni*: Public health hazards and potential control methods in poultry: A review. Vet. Med.-Czech. 49:441–446.

Higgins, S. E., J. P. Higgins, A. D. Wolfenden, S. N. Henderson, A. Torres-Rodriguez, G. Tellez, and H. Hargis. 2008. Evaluation of a *Lactobacillus*-based probiotic culture for the reduction of *Salmonella enteritis* in neonatal broiler chicks. Poult. Sci. 87:27–31.

Hochepied, T., F. G. Berger, H. Baumann, and C. Libert. 2003. α1-Acid glycoprotein: An acute protein with inflammatory and immunomodulating properties. Cytokine Growth Factor Rev. 14:25–34.

Hong, H. A., L. H. Duc, and S. M. Cutting. 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. 29:813–835.

Hong, Y. H., H. S. Lillehoj, S. H. Lee, R. A. Dalloul, and E. P. Lillehoj. 2006a. Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections. Vet. Immunol. Immunopathol. 114:209–223.

Hong, Y. H., H. S. Lillehoj, E. P. Lillehoj, and S. H. Lee. 2006b. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria maxima* infection of chickens. Vet. Immunol. Immunopathol. 114:259–272.

Huang, J. M., R. M. La Ragione, A. Nunez, and S. M. Cutting. 2008. Immunostimulating activity of *Bacillus* spores. FEMS Immunol. Med. Microbiol. 53:195–203.

Juul-Madsen, H. R., B. Viertlboeck, A. L. Smith, and T. W. F. Göbel. 2008. Avian innate immune responses. Pages 129–158 in Avian Immunology. F. Davison, B. Kaspers, and K. A. Schat, ed. Academic Press, London, UK.

Kaiser, P., and P. Stäheli. 2008. Avian cytokines and chemokines. Pages 203–222 in Avian Immunology. F. Davison, B. Kaspers, and K. A. Schat, ed. Academic Press, London, UK.

Kefali, S., and N. Y. Toker. 2006. Effects of probiotics on some acute phase proteins in broilers exposed to *Salmonella* Typhimurium lipopolysaccharides. Arch. Geflügelkd. 70:270–277.

Khaksefidi, A., and T. Ghoorchi. 2006. Effect of probiotic on performance and immunocompetence in broiler chicks. Jpn. Poult. Sci. 43:296–300.

Kirjavainen, P. V., H. S. El-Nezami, S. J. Salminen, J. T. Ahokas, and P. F. A. Wright. 1999. The effect of orally administered viable probiotic and dairy lactobacilli on mouse lymphocyte proliferation. FEMS Immunol. Med. Microbiol. 26:131–135.

Koenen, M. E., A. G. Boonstra-Blom, and S. H. M. Jeurissen. 2002. Immunological differences between layer- and broiler-type chickens. Vet. Immunol. Immunopathol. 89:47–56.

Koenen, M. E., J. Kramer, R. van der Hulst, L. Heres, S. H. M. Jeurissen, and W. J. A. Boersma. 2004. Immunomodulation by probiotic lactobacilli in layer- and meat-type chickens. Br. Poult. Sci. 45:355–366.

Lange, L. D. 2007. Do probiotics work for poultry? World Poult. 23:12–13.

Lee, B. D., A. W. Zhang, C. K. Sung, K. H. Ahn, and K. W. Lee. 2005. Effects of dietary yeast (*Saccharomyces cerevisiae*) components on growth performance and cholesterol metabolism in broiler chickens. Kor. J. Poult. Sci. 32:49–54.

Lee, S. H., H. S. Lillehoj, R. A. Dalloul, D. W. Park, Y. H. Hong, and J. J. Lin. 2007a. Influence of *Pediococcus*-based probiotic on coccidiosis in broiler chickens. Poult. Sci. 86:63–66.

Lee, S. H., H. S. Lillehoj, E. P. Lillehoj, S. M. Cho, D. H. Park, Y. H. Hong, H. K. Chun, and H. J. Park. 2008. Immunomodulatory properties of dietary plum on coccidiosis. Comp. Immunol. Microbiol. Infect. Dis. 31:389–402.

Lee, S. H., H. S. Lillehoj, D. W. Park, Y. H. Hong, and J. J. Lin. 2007b. Effects of *Pediococcus*- and *Saccharomyces*-based probiotic (MitoMax®) on coccidiosis in broiler chickens. Comp. Immunol. Microbiol. Infect. Dis. 30:261–268.

Li, S. P., X. J. Zhao, and J. Y. Wang. 2009. Synergy of *Astragalus* polysaccharides and probiotics (*Lactobacillus* and *Bacillus cereus*) on immunity and intestinal microbiota in chicks. Poult. Sci. 88:519–525.

Lillehoj, H. S., X. Ding, M. A. Quiroz, E. Bevensee, and E. P. Lillehoj. 2005. Resistance to intestinal coccidiosis following DNA immunization with the cloned 3–1E *Eimeria* gene plus IL-2, IL-15, and IFN-γ. Avian Dis. 49:112–117.

Lillehoj, H. S., C. H. Kim, C. L. Keeler Jr., and S. Zhang. 2007. Immunogenomic approaches to study host immunity to enteric pathogens. Poult. Sci. 86:1491–1500.

Lillehoj, H. S., W. Min, and R. A. Dalloul. 2004. Recent progress on the cytokine regulation of intestinal immune responses to *Eimeria*. Poult. Sci. 83:611–623.

Lillehoj, H. S., and J. M. Trout. 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to *Eimeria* parasites. Clin. Microbiol. Rev. 9:349–360.

Lowenthal, J. W., T. E. Connick, P. G. McWaters, and J. J. York. 1994. Development of T cell immune responsiveness in the chicken. Immunol. Cell Biol. 72:115–122.

Min, W., and H. S. Lillehoj. 2002. Isolation and characterization of chicken interleukin-17 cDNA. J. Interferon Cytokine Res. 22:1123–1128.

Mountzouris, K. C., P. Tsirtsikos, E. Kalamara, S. Nitsch, G. Schatzmayr, and K. Fegeros. 2007. Evaluation of the efficacy of a probiotic containing *Lactobacillus bifidobacterium, Enterococcus,* and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poult. Sci. 86:309–317.

Murata, H., N. Shimada, and M. Yoshioka. 2004. Current research on acute phase proteins in veterinary diagnosis: An overview. Vet. J. 168:28–40.

Nava, G. M., L. R. Bielke, T. R. Callaway, and M. P. Castaneda. 2005. Probiotic alternatives to reduce gastrointestinal infections: The poultry experience. Anim. Health Res. Rev. 6:105–118.

Nayebpor, M., P. Farhomand, and A. Hashemi. 2007. Effects of different levels of direct fed microbial (PrimaLac) on growth performance and humoral immune response in broiler chickens. J. Anim. Vet. Adv. 6:1308–1313.

Ng, S. C., A. L. Hart, M. A. Kamm, A. J. Stagg, and S. C. Knight. 2009. Mechanisms of action of probiotics: Recent advances. Inflamm. Bowel Dis. 15:300–310.

Novak, K., E. Davis, K. Bos, T. Rehberger, and C. Kromm. 2007. Effect of oral administration of *Lactobacillus brevis* on turkey poult performance and immune development. Poult. Sci. 86(Suppl. 1):248. (Abstr.)

Panda, A. K., S. S. Rama Rao, M. V. L. N. Raju, and S. S. Sharma. 2008. Effect of probiotic (*Lactobacillus sporogenes*) feeding on egg production and quality, yolk cholesterol and humoral immune response of White Leghorn layer breeders. J. Sci. Food Agric. 88:43–47.

Park, S. S., H. S. Lillehoj, P. C. Allen, D. W. Park, S. FitzCoy, D. A. Bautista, and E. P. Lillehoj. 2008. Immunopathology and cytokine responses in broiler chickens coinfected with *Eimeria maxima* and *Clostridium perfringens* using an animal model of necrotic enteritis. Avian Dis. 52:14–22.

Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82:627–631.

Rehberger, T. G., and D. S. Jordan-Parrott. 2005. Methods and composition for reducing *E. coli* disease and enhancing performance. US Pat. No. 7,618,640. Agtech Products Inc., assignee.

Reid, G., and R. Friendship. 2002. Alternative to antibiotic use: Probiotics for the gut. Anim. Biotechnol. 13:97–112.

Roller, M., G. Rechkemmer, and B. Watzl. 2004. Prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* modulates intestinal immune functions in rats. J. Nutr. 134:153–156.

Smith, J. A., and J. D. Helm. 2008. Report of the committee on transmissible diseases of poultry and other avian species. http://www.usaha.org/committees/reports/2008/report-pad-2008.pdf Accessed May 2009.

Subramanian, B. M., R. Sriraman, N. Hanumantha Rao, J. Raghul, D. Thiagarajan, and V. A. Srinivasan. 2008. Cloning, expression and evaluation of the efficacy of a recombinant *Eimeria tenella* sporozoite antigen in birds. Vaccine 26:3489–3496.

Takahashi, K., N. Miyake, T. Ohta, Y. Akiba, and K. Tamura. 1998. Changes in plasma α1-acid glycoprotein concentration and selected immune response in broiler chickens injected with *Escherichia coli* lipopolysaccharide. Br. Poult. Sci. 38:152–155.

Talebi, A., B. Amirzadeh, B. Mokhtari, and H. Gahri. 2008. Effects of a multi-strain probiotic (PrimaLac) on performance and antibody responses to Newcastle disease virus and infectious bursal disease virus vaccination in broiler chickens. Avian Pathol. 37:509–512.

Vila, B., A. Fontgibell, I. Badiola, E. Esteve-Garcia, G. Jimenez, M. Castillo, and J. Brufau. 2009. Reduction of *Salmonella enterica* var. Enteritis colonization and invasion by *Bacillus cereus* var. toyoi inclusion in poultry feeds. Poult. Sci. 88:975–979.

Willis, W. L., O. S. Isikhuemben, and S. A. Ibrahim. 2007. Performance assessment of broiler chickens given mushroom extract alone or in combination with probiotics. Poult. Sci. 86:1856–1860.

Willis, W. L., and L. Reid. 2008. Investigating the effects of dietary probiotic feeding regimens on broiler chicken production and *Campylobacter jejuni* presence. Poult. Sci. 87:606–611.

Yang, Y., P. A. Iji, and M. Choct. 2009. Dietary modulation of gut microflora in broiler chickens: A review of the role of six kinds of alternatives to in-feed antibiotics. World's Poult. Sci. J. 65:97–114.

Zhang, A. W., B. D. Lee, K. W. Lee, K. B. Song, G. H. Ahn, and C. H. Lee. 2005a. Effects of graded levels of dietary *Saccharomyces cerevisiae* on growth performance and meat quality in broiler chickens. Asian-australas. J. Anim. Sci. 18:699–703.

Zhang, A. W., B. D. Lee, K. W. Lee, K. B. Song, G. H. Ahn, and C. H. Lee. 2005b. Effects of yeast (*Saccharomyces cerevisiae*) cell components on growth performance, meat quality, and ileal mucosa development of broiler chicks. Poult. Sci. 84:1015–1021.